Enhanced Accumulation of Sialyl Lewis X-Carboxymethylpullulan Conjugate in Acute Inflammatory Lesion

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Purpose. E-selectin is a cell adhesion molecule that is specifically expressed in the inflammatory vascular endothelium in response to cytokines such as IL-1β and TNF-α, and interacts with specific ligands containing sialyl Lewis X (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc-, SLe^x). In order to investigate the ability of E-selectin ligands to target the inflammatory site, the tissue distribution of carboxymethylpullulan (CMPul) modified with SLe^x was studied.

Methods. CMPul conjugates with various saccharides containing SLe^x and monovalent SLe^x were intravenously administered to mice with ear edema induced by arachidonic acid, and their distributions to the inflamed ear and other tissues were studied. To determine the microdistributions of these compounds, the inflamed ear was subjected to microautoradiography.

Results. After intravenous administration $AUC_{0\text{-}24h}$ of SLe^x-CMPul, which binds to E-selectin, in the inflamed ear was about 300-fold and 2.5-fold higher than that of monovalent SLe^x and CMPul conjugated with other saccharides, which can not serve as ligands for E-selectin. Microautoradiography also revealed SLe^x-CMPul accumulated at the microvessels in the inflammatory lesions.

Conclusions. SLe^x-CMPul was found to have the potential to target drugs to the inflammatory lesion.

KEY WORDS: tissue distribution; E-selectin; sialyl Lewis X; carboxymethyl pullulan; inflammation.

INTRODUCTION

Receptor-mediated targeting of carbohydrate has been proposed as a potential method for site-specific drug delivery. One well-known example is the successful hepatic targeting using galactose-specific recognition of the asialoglycoprotein receptor (1). However, there are disadvantages with the process. For example, in the case of hepatic tumor, the anti-tumor drugs must be delivered selectively to the tumor sites as they would have unfavorable side effects if delivered to non-tumor sites. It would be ideal not to have tissue-specific but lesion-specific drug targeting to achieve enhancement of the drug actions and reduction of drug toxicity.

Recently, many cell adhesion molecules related to endothelial cell-leukocyte interactions in the immune system have been reported (2). Most play pivotal roles in recruiting leukocytes from the blood into the inflammatory sites. Here, we focused on E-selectin which mediates the initial step of binding between leukocytes and endothelial cells (2). Recently, many studies have shown that E-selectin has a calcium-dependent carbohydrate recognition domain and binds to strongly sialylated, fucosylated oligosaccharides such as SLe^x, sialyl Lewis a (SLe^a), and weakly to other oligosaccharides such as Le^x, Le^a, and Le^b (3). Furthermore, E-selectin is specifically expressed on the vascular endothelial cells stimulated by cytokines, such as IL-1β or TNF-α at inflammatory sites (2). Therefore, application of this E-selectin-SLe^x recognition system to drug targeting should result in drug delivery not to normal tissue, but directly to inflammatory lesions. However, SLe^x is thought to be rapidly filtered out at the glomerulus because of its high hydrophilicity and low molecular weight. In order to solve this problem, we introduced the SLe^x moiety to the polysaccharide CMPul. Readily prepared from pullulan (an α1-6 linear polymer of maltotriose), CMPul has a high molecular weight with weakly negative charges and is retained in the circulating blood (4). Since it has been reported that some macromolecules have the favorable property of accumulating in tissues with enhanced vascular permeability, such as cancer (5) and inflammatory sites, where E-selectin is up-regulated, a synergistic effect between SLe^x and macromolecules may also be expected.

In this work we studied the tissue distribution of mono- or oligosaccharide-CMPul conjugates in mice with ear edema induced by arachidonic acid to examine their potential for drug targeting to inflammatory sites. This serves as one of the acute phase inflammatory models. The microdistributions of the conjugates were also examined by microautoradiography.

MATERIALS AND METHODS

Syntheses of Oligosaccharides and Oligosaccharide-CMPul Conjugates

CMPul (d.s. (degree of substitution) of the carboxymethyl group: 0.6) was prepared by Nogusa's method (4) from pullulan (apparent M.W. 150,000), which was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayma, Japan). Oligosaccharides and their conjugates with CMPul were synthesized as described previously (6). Sialyl Lewis X and sialyl N-acetyllactosamine (Neu5Acα2-3Galβ1-4GlcNAc-, SLN) were synthesized by an improved method. These oligosaccharides and Neu5Ac were introduced to CMPul through hexaethyleneglycol as a spacer. The d.s. refers to the number of modified substances, such as carboxymethyl group, SLe^x, SLN, and Neu5Ac, per glucose residue in the pullulan molecule.

Radiolabeling of SLe^x and Oligosaccharide-CMPul Conjugates

The amino group of a SLe^x derivative (SLe^x-$(O\text{-}CH_2CH_2)_6$-NH_2: abbreviated as SLe^x-NH_2) was labeled with *N*-succinimidyl [2,3-^{3}H] propionate as follows. To a solution of SLe^x-NH_2 (2 mg, 1.84 μmol) in water (400 μl) were added 187 μl of 20 mM cold *N*-succinimidyl propionate (3.74 μmol), 400 μl of *N*-succinimidyl [2,3-^{3}H] propionate (3.92 nmol) in toluene (1 mCi/ml, Amersham International plc.), and 56 μl of 0.1 M *N*-methylmorpholine (5.61 μmol). After being stirred for 20 h

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at room temperature, the remaining untreated *N*-succinimidyl propionate and *N*-succinimidyl [2,3-^{3}H] propionate were removed by chloroform extraction and unreacted SLe^x-NH_2 was adsorbed to Dowe 50W × 8 (H^+). Radiolabeled SLe^x-NH_2 was purified by gel filtration chromatography on Bio-Gel P-2 (1 × 50 cm) equilibrated with 50 mM pyridine/AcOH (pH 5.0). Radioactivity and fucose contents were quantified by liquid scintillation counter and Gibbons' method (7), respectively, and radiolabeled SLe^x-NH_2 fractions were pooled.

CMPul and CMPul conjugates were labeled with [2-^{3}H] glycine as follows. To CMPul (2 mg) placed on a reaction vessel were added 200 μl of [2-^{3}H] glycine in water (1 mCi/ml, Amersham International plc.) and 2 mg of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in *N,N*-dimethyl formamide (200 μl). The reaction mixture was stirred for 12 h at room temperature and applied to a PD-10 column (Pharmacia-LKB, Uppsala, Sweden) equilibrated with saline and the void fraction was pooled.

^{125}I-labeled CMPul conjugates were prepared by the chloramine T method (8) after the introduction of tyramine.

In Vitro Binding Assay

E-selectin-immunoglobulin fusion protein (E-selectin-Ig) is a recombinant chimeric molecule containing the lectin domain, epidermal growth factor repeat and six complement regulatory repeats coupled to the hinge, CH2, and CH3 regions of human IgG1 (9). The fusion protein was prepared by protein A affinity chromatography from culture media of HEK293 cells stably transfected with cDNA encoding E-selectin-Ig, as described previously (10). Binding of E-selectin-Ig to the mucin-like ligands in cultured LS180 cells was assessed using ELISA according to the previous method with minor modifications (11). Human colon carcinoma line, LS180, obtained from American Type Culture Collection, was cultured in a 96-well plate at a density of 2 × 10^6 cells/ml in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) (GIBCO-BRL, Grand Island, New York, U.S.A.). After the incubation for 24 h at 37°C, the cells were washed 4 times with phosphate-buffered saline (PBS) and fixed with 100 μl of 2% formaldehyde in PBS for 20 min at room temperature. After washing 4 times with PBS, plates were incubated with 200 μl of 2% bovine serum albumin (BSA) in Hanks balanced salt solution (HBSS, pH 7.6) for 1 hr. After washing 3 times with HBSS, plates were incubated with 30 nM E-selectin-Ig in 1% BSA/HBSS, in the absence or presence of various concentrations with SLe^x-NH_2 or CMPul conjugates dissolved in dimethylsulfoxide for 3 hr. After washing 3 times with HBSS, the plates were incubated with peroxidase-conjugated goat anti-human IgG Fc antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, Pennsylvania, U.S.A.) in 1% BSA/HBSS. After 30-min incubation, plates were washed three times with HBSS and incubated with 50 mM sodium citrate/sodium phosphate buffer, pH 5.0, containing 0.8 mg/ml o-phenylenediamine dihydrochloride and 0.015% (v/v) hydrogen peroxide. Bound E-selectin-Ig was determined by measuring the absorbance at 490 nm after addition of 4 N H_2SO_4. Specific binding was calculated by subtracting the signal generated in the absence of E-selectin-Ig.

Tissue Distribution Experiment Using Ear Edema Model Mice

Male ICR mice (27–33 g body weight: age 5–6 weeks) were obtained from Japan SLC, Inc. and allowed free access to water and food (standard laboratory chow). Mice were anesthetized with diethyl ether. ^{3}H-labeled SLe^x or mono- or oligosaccharide (SLe^x, SLN, or Neu5Ac)-CMPul conjugates were administered intravenously at a dose of 365 nmol/kg as the concentration of saccharide. Immediately after the administration, arachidonic acid (Sigma Chemical Co., Missouri, U.S.A.) in acetone (1 mg/20 μl) was applied to both surfaces of the right ear. At various intervals after administration, the mice were anesthetized again and exsanguinated through the heart or femoral artery. The lung, spleen, kidney, liver, right ear, and left ear were then excised, rinsed with saline, and weighed. After the plasma and each tissue had been dried on a combustion cone (Packard Instrument Co., Inc., Illinois, U.S.A.) at room temperature, the ^{3}H in each sample was collected as ^3H_2O by the combustion method (Automatic Sample Combustion System, Aloka ASC-113, Tokyo, Japan). The amount of ^{3}H radioactivity was measured with a liquid scintillation counter (Aloka LSC-3500, Tokyo, Japan) using a liquid scintillation cocktail (Aquasol-II, New England Nuclear Research, Boston, Massachusetts, U.S.A.).

In inhibition experiments of tissue distribution, cold inhibitory compounds were simultaneously administered with ^{3}H-labeled conjugate at the 100-fold dose. The amount of ^{3}H radioactivity was counted as shown above.

Microautoradiography

Male ICR mice (27–33 g body weight: age 5–6 weeks) were obtained from Japan SLC, Inc., and allowed free access to water and food (standard laboratory chow). Mice were anesthetized with diethyl ether. ^{125}I-labeled SLe^x-or SLN-CMPul, with specific activities of 4.90 mCi/0.2 mg/ml and 4.20 mCi/0.2 mg/ml, respectively, were administered intravenously with a 5 ml/kg dose. Immediately after administration, arachidonic acid (Sigma Chemical Co., Missouri, U.S.A.) in acetone (1 mg/20 μl) was applied to both surfaces of the right ear. Twenty-four hours after the administration, the mice were anesthetized again and exsanguinated through the heart. The right ear and left ear were excised and rinsed with saline. These tissues were frozen by immersion in isopentane cooled with liquid nitrogen and sliced (4–6 μm thick) with a microtome. The tissue sections were exposed to Kodak light-sensitive emulsion (NTB-2) for 3 months. After developing and fixation, they were stained with hematoxylin-eosin.

Statistical Analysis

The $AUC_{0\text{-}24h}$ in each tissue was calculated and statistically compared as described previously (12). Tukey tests were performed on cold inhibition data. A minimum P-value of 0.05 was used as the significance level for all tests.

RESULTS

Structures and Physicochemical Characteristics of Mono- or Oligosaccharide-CMPul Conjugates

Table I shows the structures and physicochemical characteristics of CMPul conjugates (6). SLe^x is one of the typical

Table I. Structure and Physicochemical Characteristics of Mono- or Oligosaccharide-CMPul Conjugates

Compound	Structure of added saccharide	Apparent molecular weight	d.s.[a] of added saccharide (mol/mol Glc)	Content of added saccharide (%)
CMPul	—	190,000	—	—
Neu5Ac-CMPul	Neu5Ac α 2-	250,000	0.12	13.5
SLN-CMPul	Neu5Ac α 2-3Gal β 1-4GlcNac β 1-	300,000	0.13	27.0
SLe^x-CMPul	Neu5Ac α 2-3Gal β 1-4GlcNac β 1- 3 / Fucα 1	320,000	0.13	31.0

[a] Degree of substitution. The d.s. refers to the ratio of glucose residues, which were modified with mono- or oligosaccharides, to total glucose residues in the pullulan molecule.

E-selectin ligands. SLN and Neu5Ac, which have been reported to not bind to E-selectin (3,13), were chosen as negative controls. The apparent molecular weights of conjugates were calculated on the basis of both, the apparent molecular weight of CMPul, which was estimated by gel filtration, and the content of saccharide in the conjugates, which was determined by quantification of Neu5Ac (by resorsinol method (14)). The d.s. of the added saccharide was nearly constant.

Evaluation of Concentration-Time Profile of Monovalent SLe^x and SLe^x-CMPul in Plasma and Inflamed Ear in Ear Edema Model Mice

In order to evaluate the tissue distributions of compounds, we used the ear edema model induced by arachidonic acid, one of the acute phase inflammatory models. To confirm the expression of E-selectin, we estimated the influx of neutrophils in the inflamed ear. It increased from 2 h to 12 h and remained high until 24 h after application of arachidonic acid. When we evaluated the time course of E-selectin expression in human umbilical vein endothelial cells by ELISA, its profile correlated well with the profile of this neutrophil influx. Moreover, Hensley (15) has reported that the administration of a soluble form of human E-selectin markedly decreased the influx of neutrophils into the inflamed ear in this model, suggesting E-selectin was functionally expressed. From these findings, we thought we could estimate the compounds as homing devices to E-selectin by using this inflammatory model.

The concentrations of SLe^x-NH_2 in plasma and inflamed ear were estimated for 24 h after intravenous administration to mice with inflamed ear (Fig. 1). The plasma concentration of SLe^x-NH_2 decreased rapidly and could not be detected after an hour. Consequently, the concentration of SLe^x-NH_2 in the inflamed ear remained low. This suggests monovalent SLe^x was not suitable as a homing device for targeting E-selectin.

To enhance the potency of a CMPul carrier we introduced SLe^x moieties. This conjugation was expected to prolong the disappearance rate of SLe^x from the circulation and to bind with E-selectin with high affinity due to a multivalent effect, which is observed in members of the C-type lectin (16). Fig. 2 represents the inhibitory effect of SLe^x-NH_2 and CMPul conjugates on the binding of E-selectin-immunoglobulin fusion proteins (E-selectin-Ig) to LS180 cells, which express E-selectin ligands. The compound containing SLe^x moieties, SLe^x-NH_2 and SLe^x-CMPul, was a strong inhibitor, indicating they bound well to E-selectin, whereas SLN-CMPul or CMPul failed to demonstrate the substantial binding to E-selectin in this assay. These results showed the binding of SLe^x-CMPul was not due to nonspecific physicochemical effects of CMPul, such as negative charges, but rather reflected specific structural features of SLe^x, although the multivalent effect of SLe^x-CMPul was not observed.

The concentration-time profiles in plasma and inflamed ear after intravenous administration of SLe^x-CMPul conjugates are presented in Fig. 1. Introducing SLe^x to CMPul caused a striking enhancement of the concentration in both plasma and

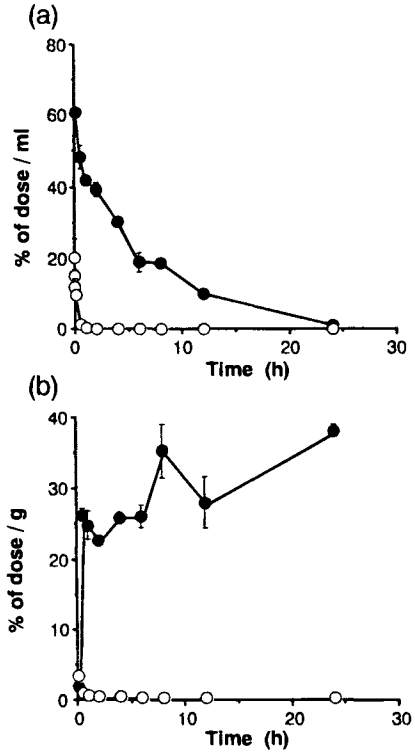

Fig. 1. Concentration-time profile of monovalent SLe^x (○) and SLe^x-CMPul (●) in plasma (a) and inflamed ear (b) after intravenous administration at a dose of 365 nmol/kg as the concentration of SLe^x in ear edema model mice. Data represent mean ± S.D. of three mice.

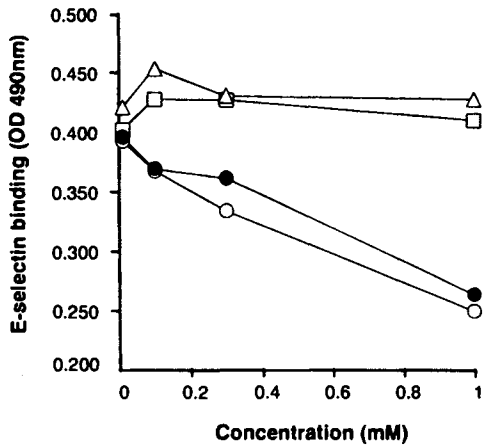

Fig. 2. Effect on binding of E-selectin-immunogobulin fusion proteins to LS180 cells. To determine inhibitory activity, SLe^x-NH_2 (●), CMPul (□), SLN-CMPul (△) or SLe^x-CMPul (○) was added to microtiter plates at the indicated concentrations. Data represent the mean of optical density measurements for duplicate wells.

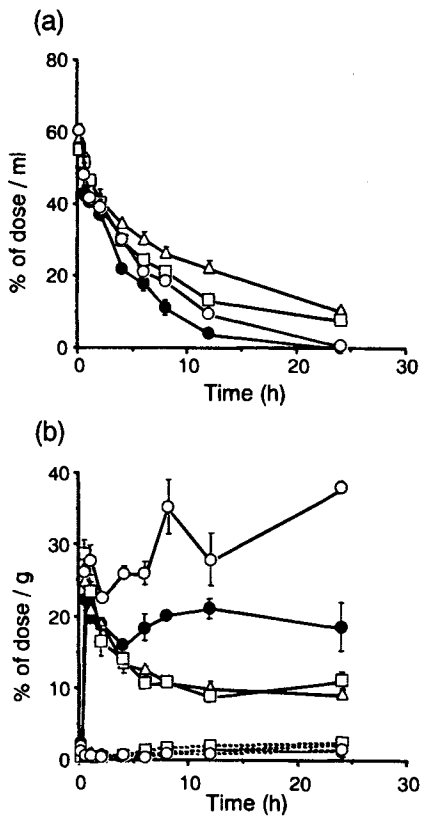

Fig. 3. Concentration-time profile of CMPul (●), Neu5Ac-CMPul (△), SLN-CMPul (□) and SLe^x-CMPul (○) in plasma (a) and inflamed (——) or non-treated (----) ear (b) after intravenous administration at a dose of 365 nmol/kg as the concentration of each added saccharide in ear edema model mice. Data represent mean ± S.D. of three mice.

inflamed ear. The AUC_{0-24h} of SLe^x-CMPul in plasma was about 40-fold greater than that of monovalent SLe^x. In the inflamed ear, the enhanced vascular permeability caused the initial rapid distribution of SLe^x-CMPul. After a slight decrease, the amount of SLe^x-CMPul gradually increased from 4 h to 24 h again. This accumulation of SLe^x-CMPul was explained by E-selectin expression after the stimulation of endothelial cells by inflammatory cytokines. The AUC_{0-24h} in inflamed ear of SLe^x-CMPul was about 300-fold greater than that of monovalent SLe^x (Table II).

Comparison of Mono- or Oligosaccharide-CMPul Conjugates in the Inflamed Ear in Ear Edema Model Mice

In order to estimate the influence of SLe^x on the accumulation of SLe^x-CMPul in the inflamed ear, the distributions of SLe^x-CMPul were compared with those of CMPuls modified with mono- or other oligosaccharides which can not bind to E-selectin (Fig. 3). Every conjugate tended to accumulate in the inflamed ear due to a prolonged circulation time. However, the concentrations of conjugates in inflamed ear did not correlate with their plasma concentrations. For example, the AUC_{0-24h} of SLe^x-CMPul was 2.5-fold higher than that of both Neu5Ac-CMPul and SLN-CMPul in spite of its showing a lower plasma concentration (Fig. 3, Table II). The accumulation of SLe^x-CMPul in the inflamed ear would be due to its ability to bind to E-selectin (Fig. 2).

The concentration of CMPul, which did not bind to E-selectin as well as SLN-CMPul, as shown in Fig. 2, was lower than that of SLe^x-CMPul in the inflamed ear, but higher than that of Neu5Ac- and SLN-CMPul. This could be interpreted

Table II. AUC_{0-24h} in Each Tissue of SLe^x-NH_2 and Mono- or Oligosaccharide-CMPul Conjugates Administered Intravenously at a Dose of 365 nmol/kg as the Concentration of Saccharide in Ear Edema Model Mice

	$AUC_{0-24h,\ tissue}$ (% of dose·h/ml or g)						
Compound	Plasma	Right ear (inflammation)	Left ear	Lung	Spleen	Kidney	Liver
SLe^x-NH_2	9.7	2.1	1.7	1.5	0.8	8.1	1.0
CMPul	275.9	468.0[a]	27.2	48.6	548.8	42.8	342.6
Neu5Ac-CMPul	588.9	276.8	33.6	67.5	71.2	56.2	250.5
SLN-CMPul	463.4	278.4	43.1	51.2	82.2	58.1	308.0
SLe^x-CMPul	368.5	689.9[a]	24.6	63.6	1339.3	37.1	264.7

Note: Each data represents mean (n = 3).
[a] Significantly different from the corresponding values of other CMPul conjugates ($p < 0.01$).

as a biological response of macrophages for the following reasons. The first, is the concentration of CMPul in the inflamed ear gradually increased from 6 h after administration in the concentration-time profile (Fig. 3). This would be consistent with the fact that macrophages accumulate in inflammatory sites about 6 h after the induction of inflammation (17). Secondly, work in our laboratory has pointed out thioglycolate-stimulated peritoneal macrophages took up CMPul through scavenger receptors in vitro (unpublished data). These findings suggest CMPul accumulation in the inflamed ear is affected by the uptake of activated macrophages. When 100-fold molar cold succinyl HSA, one of the ligands for scavenger receptors (18), CMPul or Neu5Ac-CMPul, was co-administrated in the tissue distribution experiment, the accumulation of CMPul in the inflamed ear at 24 h after administration was markedly blocked by cold succinyl HSA and CMPul, falling to the levels observed on administration of Neu5Ac- or SLN-CMPul alone (Fig. 4), whereas Neu5Ac-CMPul could not decrease the CMPul level. On the other hand, none of the cold inhibitors decreased the SLe^x-CMPul level, indicating SLe^x-CMPul differed from Suc-HSA, CMPul, and Neu5Ac-CMPul in the mechanism of accumulation in the inflamed ear and activated macrophages did not take up SLe^x-CMPul through scavenger receptors in vivo. The same results were found at 6 h after administration (data not shown). This suggested the concentration of CMPul in the inflamed ear considerably increased as a result of uptake by macrophages, which would not contribute to the accumulation of other conjugates in the inflamed ear. If the uptake by the macrophages is subtracted, the original level of CMPul in the inflamed ear should be equal to that of Neu5Ac- or SLN-CMPul.

These findings show CMPul or saccharide-modified CMPul conjugates tend to accumulate in the inflammatory site due to their prolonged circulation time, and additional properties of SLe^x in SLe^x-CMPul resulted in 2.5-fold higher AUC_{0-24h} in the inflamed ear than CMPul or other conjugates.

We predicted the microdistribution of SLe^x-CMPul would be distinguishable from those of other conjugates because E-selectin is expressed on the cell surface of endothelial cells at inflammatory sites. To confirm this, we used microautoradiography of the radiolabeled conjugates in the ear edema model mice. ^{125}I-radiolabeled SLe^x-CMPul and SLN-CMPul were intravenously administered just before application of arachidonic acid and microscopic distribution in the inflamed ear at 24 h after administration was evaluated (Fig. 5). Many Ag grains of SLe^x-CMPul were observed along with microvessels, whereas only a few grains were widespread with administration of SLN-CMPul. This trend was more marked at 24 h than 6 h after administration (data not shown).

These results suggest that SLe^x-CMPul would be superior to other CMPul conjugates in both quantity and specificity for targeting microvessels in the inflammatory sites.

DISCUSSION

In this study we demonstrate CMPul conjugating with SLe^x is a novel carrier for targeting a drug specifically to microvessels in the inflammatory sites. This system would be useful for inflammations, which are found in a wide array of human diseases. Many immunohistochemical approaches have revealed E-selectin is expressed in lesions in atherosclerosis, vasculitis, ischemia, and reperfusion injury, sepsis, adult respiratory distress syndrome, asthma, arthritis, graft rejection, and some kinds of tumors (19,20).

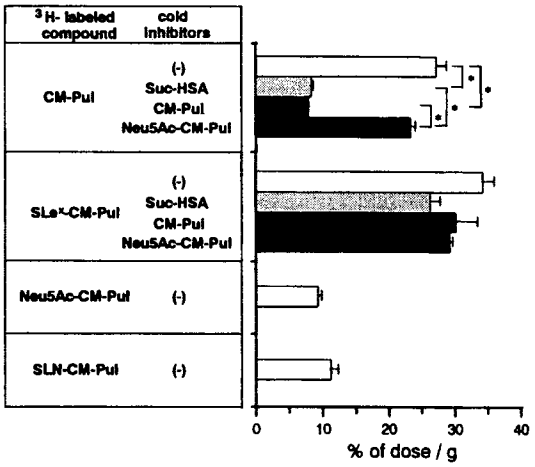

Fig. 4. Inhibitory effects of cold succinyl HSA (▨), CMPul (▨) and Neu5Ac-CMPul (■) on the accumulation of CMPul and mono-or oligosaccharide-CMPul conjugates in inflamed ear. ^{3}H-labeled compound was intravenously co-administered with each cold inhibitor at a dose of 365 nmol/kg or 36500 nmol/kg as the concentration of each saccharide. Radioactivities at 24 h after administration were estimated. The control, which was administered the radiolabeled compound alone, is shown by the open column. Data represent mean (±) S. D. of three mice. * $p < 0.01$.

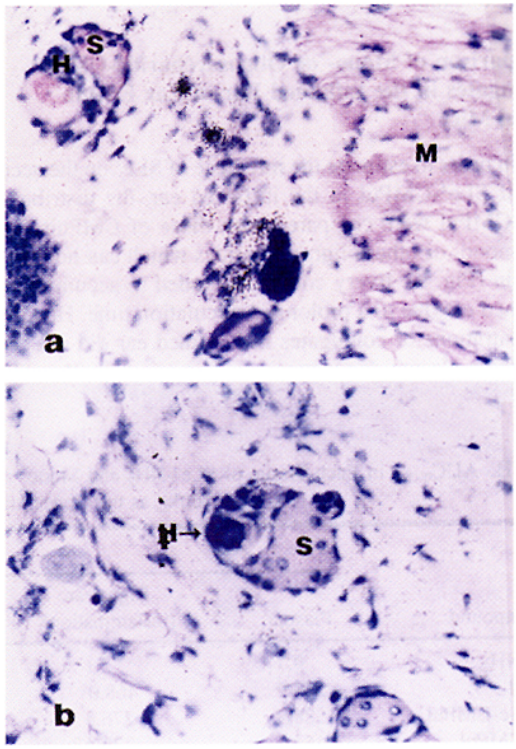

Fig. 5. Microautoradiographs of the inflamed ear obtained at 24 h after intravenous administration of ^{125}I-labeled SLe^x-CMPul (a) and SLN-CMPul (b) at a dose of 1 mg/kg (H, hair follicle; M, cutaneous muscle; S, subaceous glands).

After intravenous administration, SLe^x-NH_2 was rapidly filtered out at the glomerulus, as it is hydrophilic and a small molecule. In order to improve the disposition characteristics of SLe^x-NH_2, SLe^x moieties were introduced to CMPul. This was thought to be a favorable approach, because, CMPul with a high molecular weight of larger than 50,000 and negative charges, is not susceptible to glomerular filtration and the d.s. of carboxymethyl group in CMPul was optimized to avoid, to some extent, its accumulation in the liver (unpublished data). These physicochemical characteristics of CMPul caused the higher plasma concentration and higher accumulation of SLe^x-CMPul in the inflammatory lesions (Fig. 3, Table II). The AUC_{0-24h} of SLe^x-CMPul in the inflamed ear was 300-fold larger than that of SLe^x-NH_2.

SLe^x-CMPul was compared with other SLe^x-modified polysaccharides, such as carboxymethylchitosan (CMCht, apparent molecular weight 100,000) or desulfated heparin (DSH, apparent molecular weight 40,000). AUC_{0-24h} of SLe^x-CMPul in plasma was about 3-fold and 32-fold greater than that of SLe^x-CMCht and SLe^x-DSH, respectively (data not shown). The reasons for these differences were: (1) SLe^x-CMCht and SLe^x-DSH are smaller than SLe^x-CMPul; (2) SLe^x-CMCht is sensitive to lysozyme in the circulation; (3) SLe^x-DSH is susceptible to glomerular filtration. According to the plasma level, AUC_{0-24h} of SLe^x-CMPul in the inflamed ear was about 6-fold and 13-fold larger than that of SLe^x-CMCht and SLe^x-DSH, respectively (data not shown). These findings showed SLe^x-modified polysaccharides are extremely useful carriers for enhancing accumulation in inflamed lesions and SLe^x-CMPul has the best disposition characteristics among them.

However, this accumulation of SLe^x-CMPul is due not only to its physicochemical characteristics but also to its biological characteristics. To date, many studies of E-, P-, and L-selectin have been directed toward therapeutic inhibitions based on ligands and ligand analogues (21). Regarding binding strength, most of monomeric ligands possess the IC_{50} values (rough indicator of binding constant) in the μM-mM range, whereas some of multivalent ligands have the binding strength in the nM-μM range (22). In this study, we examined the binding strength of SLe^x-CMPul in binding assay using the E-selectin-immunoglobulin fusion proteins (E-selectin-Ig). Although SLe^x-CMPul suppressed the binding of E-selectin-Ig, its binding strength was almost the same as that of SLe^x-NH_2, demonstrating little multivalent effect of SLe^x-CMPul. This might be due to the differences in the backbone structures of the SLe^x-carrying macromolecules. Most multivalent SLe^x bind to the polypeptide backbone such as bovine serum albumin that is prolate ellipsoidal molecule (23) and might cluster SLe^x in the favorable configuration, whereas pullulan might not create SLe^x multivalency for its linear conformation.

In tissue distribution studies of CMPul conjugates, the AUC_{0-24h} of SLe^x-CMPul in the inflamed ear was 2.5-fold larger than that of Neu5Ac-CMPul or SLN-CMPul. The differences in their distributions may not have been due to a nonspecific physicochemical effect but rather may have reflected differences in the ability to bind to E-selectin, because they all had similar physicochemical characteristics, such as the molecular weight, d.s. of the mono- or oligosaccharide and a negative charge. All of these findings clearly demonstrated SLe^x-CMPul accumulated at the inflammatory site due to synergistic effects, arising from its physicochemical and biological properties.

Besides the macromolecule conjugate, these beneficial properties could also be obtained from the liposome, which is thought to be a useful carrier for the multivalent form of SLe^x. Indeed, the liposome containing glycolipids with SLe^x seemed to strongly bind to E-selectin *in vitro*. Saiki (24) showed SLe^x-modified liposome blocked tumor metastasis by inhibiting the binding between B16-BL6 expressing SLe^x and endothelial cells stimulated by inflammatory cytokines. This result shows that SLe^x-modified liposome would be efficiently recognized by selectins and be useful, not only as a drug carrier, but also as an anti-inflammatory agent. However, when SLe^x-modified liposome is utilized as a drug carrier to the inflammatory site, the reticuloendothelial system (RES) must be avoided. Fortunately, some kinds of liposomes, which evade the RES and display prolonged circulation time in the bloodstream, have been developed. For example, the liposomes modified with polyethylene glycol (25), natural glycolipids (such as the monosialylganglioside, GM1 (26)) or synthetic glycolipids (such as the sialic acid derivative (27)). These technologies will be important for enhancing the accumulation of SLe^x-modified liposome in the inflammatory site.

In the present study we discovered SLe^x-CMPul accumulated in the spleen. The amount of ^{125}I-labeled SLe^x-CMPul in the spleen was strongly inhibited by co-administration of 100-fold molar cold SLe^x-CMPul but not SLN-CMPul (unpublished data), indicating this accumulation occurs in a SLe^x-specific manner. Because the accumulation of SLe^x-CMPul in the spleen was also observed in non-treated normal mice (unpublished data), the molecule responsible for this accumulation may be constitutively expressed. Also, E-selectin mRNA is reported not to be expressed in the non-inflammatory spleen (28), suggesting E-selectin would not be due to this accumulation. In order to prevent the accumulation of SLe^x-CMPul in the spleen and to increase that in the inflamed ear, the molecule causing this accumulation must be identified and its binding characteristics should be clarified.

When the E-selectin-SLe^x recognition system is used for drug targeting to the inflammatory site, the fate of E-selectin expressed on the endothelial cells is very important. Kuijpers (29) has reported the induced E-selectin molecules were rapidly removed from the surface of the endothelial cells by an active process of internalization and accumulated in an intracellular tublo-vesicular compartment with lysosomal properties. Smeet (30) has shown E-selectin was rapidly degraded in an acidic compartment after being internalized and the surface-expressed E-selectin was not down-regulated by addition of SLe^x. From these findings and the results from our microautoradiograpy study, SLe^x-CMPul may be co-internalized with E-selectin and partially degraded in an acidic compartment. Accordingly, this drug targeting system might be suitable for drugs acting on endothelial cells or their surroundings in inflammatory sites.

In conclusion, we have demonstrated that SLe^x-CMPul would be an excellent carrier for targeting a drug to an inflammatory lesion. We are currently studying the therapeutic effect of SLe^x-CMPul conjugated with a drug in an animal model.

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